Effects of Vincristine in Combination with Methotrexate and Other Antitumor Agents in Human Acute Lymphoblastic Leukemia Cells in Culture

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ABSTRACT

The effects of vincristine (VCR) in combination with methotrexate (MTX) and other antitumor agents were evaluated by cell growth inhibition assay using a human acute lymphoblastic leukemia cell line (MOLT-3). The data were analyzed with the aid of an isobologram using the concept of an envelope of additivity (G. G. Steel and M. J. Peckman, Int. J. Radiat. Oncol., 5: 85-91, 1979). Simultaneous exposure of VCR and MTX produced subadditive or mutually protective interactions. Sequential exposure to VCR followed immediately by MTX produced similar interactions. When the interval of VCR exposure first followed by then MTX was increased from 0 to 3, 8, and 24 h, the inhibitory effect of the combination moved from protection and subadditivity to additivity only. The reversed order of exposure to the 2 drugs produced an entirely different picture. Thus, when the interval of MTX exposure first followed by VCR increased from 0 to 3, 8, and 24 h, the inhibitory effects of the combination changed progressively from the area of subadditivity to the area of supraadditivity. When these data were analyzed using median effect plot analyses (T-C. Chou and P. Talalay, In: New Avenues in Developmental Cancer Chemotherapy, pp. 36-64. Orlando, FL: Academic Press, 1987), strongly synergistic interaction of this sequence at space intervals was confirmed. These data show that the synergistic effects were produced only when MTX was followed 8 or 24 h later by VCR. Other schedules were only additive or even antagonistic.

Simultaneous exposure of VCR with daunorubicin, 1-ß-D-arabinofuranosylcytosine, or bleomycin also had subadditive and protective effects. VCR, followed by daunorubicin with the interval of 24 h and vice versa, was again subadditive and protective. VCR, followed by 1-ß-D-arabinofuranosylcytosine with the interval of 24 h and vice versa, was again subadditive or additive only. Simultaneous and continuous exposures of VCR with vinblastine or l-asparaginase were only marginally supraadditive.

INTRODUCTION

The combination of VCR with MTX or other antitumor agents has been widely used for the treatment of various types of malignant diseases in humans. The administration of VCR prior to or together with MTX enhanced the steady level of intracellular MTX and decreased the efflux of MTX in animal and human tumor cells (1-6). VCR was also reported to enhance intracellular polyglutamate formation of MTX (7). These biochemical observations favor the administration of VCR prior to or simultaneously with MTX in patients with cancer. Indeed, therapeutic synergism was reported with this combination for the treatment of mice bearing L1210 leukemia (6). On the basis of these observations, administration of VCR prior to high-dose MTX was incorporated into clinical protocols (8, 9). In further studies, however, other investigators failed to demonstrate the therapeutic synergism of the VCR-MTX combination when simultaneously or sequentially administered in this order using the same L1210 system (2, 10, 11). Therefore, the therapeutic advantage of the VCR-MTX combination is at best controversial.

To evaluate the effect of drug combinations in both in vivo and in vitro studies, the concepts of synergy, additivity, and antagonism have been widely used. However, the concept of additivity which is the basis of the definition of synergy and antagonism has been used in a less precise manner. Workers have simply added the effects of the constituents used separately. Synergy, additivity, and antagonism were then deemed to be present if the effect of the drug combinations were, respectively, more than equal to or less than the sum of the effects of the constituents. If the dose-response curves of two agents follow closely to first-order kinetics, the drug combination can be evaluated by this method. However, dose-response curves do not always follow first-order kinetics, and in such cases the method described above is not suitable for the evaluation of the effect of drug combinations (12-16). To solve this problem, Steel (14, 15) proposed a method for analyzing the effects of combined radiotherapy-chemotherapy with nonlinear dose-response curves. We applied this method for the evaluation of the effects of the VCR combination with MTX and other antitumor agents in vitro.

After the present work was completed (17), we came across a newer analysis for the combined effects of 2 drugs, median effect plot analysis, developed by Chou and Talalay and increasingly used in recent years (18-20). A portion of our data were reanalyzed using this new concept.

MATERIALS AND METHODS

Cell Line

A human acute lymphoblastic leukemia cell line, MOLT-3 (21), was maintained as a suspension in culture flasks (No. 3024; Falcon Plastics, Oxnard, CA) containing RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated FBS (GIBCO) and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml; Charles Pfizer, Brooklyn, NY).

Chemotherapeutic Agents

VCR sulfate and VLB sulfate were obtained from Eli Lilly, Indianapolis, IN; MTX, Lederle Laboratories, Pearl River, NY; DNR, Farmitalia, Milan, Italy; ara-C, Upjohn, Kalamazoo, MI; BLEO sulfate, Bristol Laboratories, Syracuse, NY; ASN-ase, Merck Sharp & Dohme, West Point, PA; and TMQ through the National Cancer Institute, Bethesda, MD.

Cell Growth Inhibition by Combined Chemotherapeutic Agents

Biological effects of the drugs were evaluated by cell growth inhibition assay (22, 23). Logarithmically growing cells were harvested from the medium and resuspended to a final concentration of 1 x 10⁵ cells/ml of fresh medium.

For the continuous mode of exposure to 2 drugs, cell suspensions...
The total area enclosed by these 3 lines conceivably represents additive response or an envelope of additivity (Fig. 1).

With combinations of graded doses of Drug A and a chosen dose of Drug B, a single dose-response curve can be drawn. When the experimental ID_{50} concentration in this combination falls left of the envelope (Fig. 1, Point Pa), the 2 drugs have supraadditive interaction. When the experimental data point is within the envelope, this combination is considered to be noninteractive (additive) (Fig. 1, Point Pb). When this point is in the area right of the envelope, but within the square produced by 1.0 of the ordinate and abscissa, the drugs have subadditive interaction (Fig. 1, Point Pc). When the point is outside the square, both drugs are considered to be mutually protective (Fig. 1, Point Pd).

We find that isoeffect curves had a reasonable amount of variation from experiment to experiment when repeated. We chose to present a representative isobologram after confirmation that, in repeated experiments, individual combination data points were reproducibly in the same area of supraadditivity, additivity, or subadditivity irrespective of the envelope being lean or obese.

**Median Effect Plot Analyses**

The combined effects of 2 drugs in terms of synergism, summation, or antagonism can also be analyzed by the median effect plot (18–20). If the observed effect of 2 drugs acting simultaneously is larger or smaller than that calculated from the product expression, it is assumed that synergism or antagonism, respectively, has occurred. This method involves plotting dose-effect curves for each drug and for one or more multiple-dilution combinations of the drugs using the “median effect” equation

\[ \frac{fa}{fu} = \left( \frac{D}{ID_{50}} \right)^n \]

where \( D \) is the dose, \( ID_{50} \) is the dose required for 50% effect (e.g., 50% inhibition of cell growth at 72 h as compared to drug-free control), \( fa \) is the fraction affected by the dose \( D \), \( fu \) is the fraction unaffected, and \( m \) is a coefficient signifying the sigmoidicity of the dose-effect curve. The dose-effect curve was plotted using a logarithmic conversion of this equation which determines the \( m \) and \( ID_{50} \) values. Based on the slope of the dose-effect curves, it can be decided whether the agents have mutually exclusive effects (e.g., similar mode of action) or mutually nonexclusive effects (e.g., independent mode of action). A CI was then determined using the equation

\[ CI = \frac{(D_1)}{(D_1)^h + (D_2)^h + \alpha(D_1)(D_2)^h} \]

where \( (D_1) \) is the dose of Drug 1 required to produce x% effect alone, \( (D_2) \) is the dose that drug required to produce the same x% effect in combination with \( (D_1) \). Similarly, \( (D_3) \) is the dose of Drug 2 required to produce x% effect alone, and \( (D_4) \) is the dose required to produce the same effect in combination. If the drugs are mutually exclusive, then \( \alpha = 0 \); if mutually nonexclusive, then \( \alpha = 1 \). When \( CI = 1 \), the interaction is considered additive; when \( CI < 1 \), synergism is indicated, and when \( CI > 1 \), antagonism is indicated. Using this method, we analyzed the data presented in Fig. 3 below.

**RESULTS**

**VCR-MTX Combination.** For the simultaneous and continuous exposure of cells to VCR and MTX, the data points fell on the right side of the envelope (Fig. 2A). Certain data points were in the area of subadditivity, and others were in the area of protection. These observations can be interpreted to show that simultaneous exposure to VCR and MTX produced subadditive or mutually protective interactions. The combination of VCR exposure for 5 h followed by continuous exposure to MTX (Fig. 2B) or simultaneous 5-h exposures to VCR and MTX (Fig. 2C) produced similar interactions, subadditivity and pro-

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**Fig. 1.** An envelope of additivity is constructed from the dose-response curve of 2 drugs (A and B). If experimental data points \( Pa, Pb, Pc, \) and \( Pd \) fall in the area specified in the diagram, the 2 drug interactions can be classified as supraadditive, additive, subadditive, and mutually protective, respectively (14, 15).

For the short-term mode of exposure, the cell suspensions were dispensed into tissue culture tubes (No. 3033; Falcon) and exposed to 2 drugs with various concentrations in 95% air/5% CO\(_2\) at 37°C for 72 h. At the end of incubation, the cells were washed twice with fresh medium plus 10% FBS and were resuspended in fresh medium plus 10% FBS. The cell suspensions were then incubated for 0, 3, 8, and 24 h. At the end of the incubation period, the cells were exposed to the second drug for 5 h, washed twice, and resuspended into the same medium with 10% FBS. The cell suspensions were again incubated further for 72 h. Viable cell growth was determined by the trypan blue exclusion method. The dose-response curve was plotted on a semilog scale as a percentage of control, the cell number of which was obtained from samples with no drug exposure, but processed simultaneously.

The experiments were carried out in duplicate and repeated twice or more.

**Isobologram Analyses**

Analyses of drug interactions were performed by constructing “an envelope of additivity” (14, 15) on an isobologram as described previously (22). Based on available dose-response curves, we analyzed the combined effect of 2 drugs at the point of ID_{50}. Three isoeffect curves were drawn as follows.

**Mode I Line.** When the dose of Drug A is chosen there remains an increment in effect to be produced by Drug B. If 2 drugs were to act independently, the addition is performed by taking the increment in doses, starting from zero, that give log survivals which add up to ID_{50}.

**Mode II (A) Line.** When the dose of Drug A is chosen, an isoeffect curve can also be calculated by taking the dose increment of Drug B that gives the required contribution to the total effect up to the limit, in this case, ID_{50}.

**Mode II (B) Line.** Similarly, when the dose of Drug B is chosen, an isoeffect curve can be calculated by taking the dose increment of Drug A that gives the required contribution to ID_{50}.

When the dose-response curve of Drug A follows first-order kinetics, Mode II (B) line will be identical to Mode I line, and vice versa. When both drugs follow first-order kinetics all three isoeffect lines will converge to make a straight line connecting 1.0 of the ordinate and abscissa.
VCR AND MTX IN COMBINATION IN VITRO

Fig. 2. Isobologram of VCR in combination with MTX. A, continuous and simultaneous exposure to VCR and MTX; B, short-term exposure (5 h) to VCR followed by continuous exposure to MTX; C, short-term, simultaneous exposure to VCR and MTX; D, short-term exposure to VCR followed by short-term exposure to MTX without interval; E, same as in D except with a 3-h interval; F, same as in D except with an 8-h interval; G, same as in D except with a 24-h interval; H, short-term exposure to MTX followed by short-term exposure to VCR without interval; I, same as in H except with a 3-h interval; J, same as in H except with an 8-h interval; K, same as in H except with a 24-h interval.

Fig. 3. Dose-response curves in VCR-MTX combination. A, short-term (5 h) simultaneous exposure to VCR and MTX; B, short-term exposure to MTX followed by short-term exposure to VCR with a 3-h interval; C, short-term exposure to MTX followed by short-term exposure to VCR with a 24-h interval. MTX concentrations (M) for each symbol are shown in A. VCR concentrations (M) in the abscissa are to be divided by 10\(^4\) to give the actual value studied.

Sequential exposure of 2 drugs, however, produced interval-dependent changes in the 2-drug interaction. Thus, when the cells were exposed to VCR for 5 h, followed by MTX for 5 h, the data moved from the areas of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively). The interval-related changes in the 2-drug interaction were more pronounced when sequential exposure to the 2 drugs was reversed. Thus, when cells were exposed to MTX for 5 h, immediately followed by exposure to VCR for 5 h, the data points were in the area of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively). The interval-related changes in the 2-drug interaction were more pronounced when sequential exposure to the 2 drugs was reversed. Thus, when cells were exposed to MTX for 5 h, immediately followed by exposure to VCR for 5 h, the data points were in the area of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively). The interval-related changes in the 2-drug interaction were more pronounced when sequential exposure to the 2 drugs was reversed. Thus, when cells were exposed to MTX for 5 h, immediately followed by exposure to VCR for 5 h, the data points were in the area of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively). The interval-related changes in the 2-drug interaction were more pronounced when sequential exposure to the 2 drugs was reversed. Thus, when cells were exposed to MTX for 5 h, immediately followed by exposure to VCR for 5 h, the data points were in the area of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively). The interval-related changes in the 2-drug interaction were more pronounced when sequential exposure to the 2 drugs was reversed. Thus, when cells were exposed to MTX for 5 h, immediately followed by exposure to VCR for 5 h, the data points were in the area of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively). The interval-related changes in the 2-drug interaction were more pronounced when sequential exposure to the 2 drugs was reversed. Thus, when cells were exposed to MTX for 5 h, immediately followed by exposure to VCR for 5 h, the data points were in the area of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively). The interval-related changes in the 2-drug interaction were more pronounced when sequential exposure to the 2 drugs was reversed. Thus, when cells were exposed to MTX for 5 h, immediately followed by exposure to VCR for 5 h, the data points were in the area of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively). The interval-related changes in the 2-drug interaction were more pronounced when sequential exposure to the 2 drugs was reversed. Thus, when cells were exposed to MTX for 5 h, immediately followed by exposure to VCR for 5 h, the data points were in the area of subadditivity and protection at the 0-h interval (Fig. 2D) into the envelope of additivity with the interval of 3, 8, and 24 h (Fig. 2, E, F, and G, respectively).

Examples of the dose-response curve, which were the basis for constructing an isobologram of subadditivity (Fig. 2C), additivity (Fig. 2F), and supraadditivity (Fig. 2K), are shown in Fig. 3, A, B, and C, respectively.

VCR-DNR Combination. Simultaneous and continuous exposure to VCR and DNR produced subadditive effects (Fig. 4A). The effects of simultaneous 5-h exposure to the 2 drugs were subadditive or mutually protective (Fig. 4B). Sequential exposure to 2 drugs with a 24-h interval produced essentially identical interactions whether VCR preceded DNR or vice versa (Fig. 4, C and D).

VCR-ara-C Combination. The simultaneous continuous or simultaneous 5-h exposure to VCR and ara-C resulted in subadditive or protective interaction (Fig. 4, E and F). Five-h exposure to VCR followed by 5-h exposure to ara-C with the interval of 24 h produced the combination data points within the envelope of additivity (Fig. 4G). The reversed order of exposure in similar experiments moved the data points into the area of marginal subadditivity, not far from additivity (Fig. 4H).

VCR Combination with TMQ, VLB, ASN-ase, or BLEO. All the studies were done as simultaneous and continuous exposure to 2 drugs. VCR combinations with TMQ (Fig. 5A) or BLEO (Fig. 5D) were subadditive and protective, while VCR combinations with VLB (Fig. 5B) and ASN-ase (Fig. 5C) tended to be supraadditive, albeit, again not far from the area of additivity.

Median Effect Plot Analysis. In order to evaluate the rela-
Fig. 4. Isobologram of VCR in combination with DNR or ara-C. A, continuous and simultaneous exposure to VCR and DNR; B, simultaneous short-term (5 h) exposure to VCR and DNR; C, short-term exposure to VCR followed by short-term exposure to DNR with a 24-h interval; D, short-term exposure to DNR followed by short-term exposure to VCR with a 24-h interval; E, continuous and simultaneous exposure to VCR and ara-C; F, short-term simultaneous exposure to VCR and ara-C; G, short-term exposure to VCR followed by short-term exposure to ara-C with a 24-h interval; H, short-term exposure to ara-C followed by short-term exposure to VCR with a 24-h interval.

DISCUSSION

Linguistically speaking, supraadditivity, additivity, subadditivity, and mutually protective interaction used by Steel should correspond to a more common terminology of synergism, summation, antagonism, and strong antagonism, respectively, used by Chou. Since analytical methods and definitions used by these 2 workers are different, however, results obtained by these 2 methods do not necessarily fall within each respective category. Accordingly, we used each word as defined and used by the 2 respective workers. We observed that data obtained by the 2 methods gave a reasonable degree of proximity in judging synergism, summation, and antagonism.

Our studies show that the inhibitory effects of VCR in combination with MTX are schedule dependent. Exposure to VCR prior to or together with MTX resulted in additive and protective interactions (antagonism), indicating that the effects of the 2 drugs in combination are less than those obtained when either drug is used alone. Only the sequence of MTX exposure first followed by VCR, with sufficient intervals, was supraadditive (synergistic). Progressively stronger synergism of this sequence with increasing interval was confirmed by the median effect plot analysis. Our in vitro observations are consistent with reports by Chello et al. that delay of VCR administration for 24 h after MTX did produce increased survival in mice bearing L1210 leukemia (10, 11).

As the biochemical rationale of the VCR-MTX combination, VCR-induced enhancement of steady-state levels of intracellular MTX, decreased MTX efflux, and increased intracellular MTX polyglutamation were described (1-7). These reports are in contrast to observations made by Bruckner et al. who reported suppressive effects of VCR on MTX-induced inhibition of DNA synthesis (24). Our data are consistent with the findings of Bruckner et al.

The reasons why exposure to MTX followed by VCR produced synergistic interaction cannot readily be explained. No biochemical data are available as to whether MTX enhances...
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Table 1 Median effect plot analysis of data presented in Fig. 3

Data values for the combination are presented as $\frac{fa}{f}$ (fractional inhibition), CI for mutually nonexclusive assumption, and CI for mutually exclusive assumption, numbered in parentheses in this order.

### Simultaneous exposure to MTX and VCR

<table>
<thead>
<tr>
<th>MTX ($\times 10^{-6}$ M)</th>
<th>VCR ($\times 10^{-6}$ M)</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>0.1</td>
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<td>0.3</td>
<td>0.37</td>
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<tr>
<td>1.0</td>
<td>0.39</td>
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<tr>
<td>3.0</td>
<td>0.72</td>
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<tr>
<td>10</td>
<td>0.89</td>
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<td>30</td>
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| Data values for the combination are presented as $\frac{fa}{f}$ (fractional inhibition), CI for mutually nonexclusive assumption, and CI for mutually exclusive assumption, numbered in parentheses in this order. |

VCR uptake and/or binding to microtubules.

We find simultaneous exposure of VCR-MTX, VCR-ara-C, and VCR-BLEO, all of which are a part of clinically active multidrug combinations, to be not more than additive in vitro. The VCR-DNR combination was shown to be subadditive and mutually protective in vitro, whereas the same combination was reported to be therapeutically synergistic in mice bearing L1210 leukemia and in rats bearing L5222 leukemia (25, 26). Furthermore, while our study tends to show that somewhat supraadditive interaction of VCR-ASN-ase in vitro, this combination was reported to be clinically more neurotoxic (27). Possible explanations for the discrepancy between in vivo and in vitro data include: (a) target cells are different in each experimental system; (b) data from in vivo experiments take into account both therapeutic and toxic effects, while data from in vitro experiments at the cellular level give effects that will be either therapeutic or toxic depending upon the target cells used; (c) pharmacokinetic values are different between in vivo and in vitro.

**Numbers with underscore, antagonistic interactions.**

**Numbers in bold face, synergistic interactions.**
We find clinically active drug combinations do not necessarily produce supraadditivity or synergism in vitro. Conversely, synergistic combination in vitro may not always be therapeutically more efficacious. Nevertheless, our in vitro observations of supraadditivity (synergism) with the sequence of MTX followed by VCR, supported by experimental animal data (10, 11), favor the exploration of this sequence for the treatment of VCR- and MTX-sensitive human neoplasms. Similarly, VCR plus VLB may at least be additive when combined. Neuropathy is the most frequent and dose-limiting toxicity of VCR, whereas the dose-limiting toxicity of VBL is myelosuppression. The combination of VCR and VLB is tempting whether it lessens the neurotoxicity or enhances therapeutic efficacy in the treatment of patients with Vinca alkaloid-sensitive neoplasms.

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